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PROGRESS REPORT ABSTRACT

Multiple copies of estrogen responsive elements, EREs, located in the regulatory regions of highly estrogen responsive genes, synergistically regulate their gene expression. We and others previously reported that the number, location and spacing of multiple EREs influence the extent of synergy at a given promoter. Based on a model that protein-protein interactions mediate the synergy at multiple EREs, we have begun to dissect the estrogen receptor, ER alpha, to identify a region in the receptor that is directly involved in the synergy function. Previous reports have demonstrated that ER activation functions AF-1 and AF-2, located at the N- and C-terminus, respectively, of ER are specific to cell and promoter contexts. We therefore used a minimal TATA box promoter to analyze synergy mediated by ER, in the absence of other transcription factors. In COS-1 cells, AF-1 of ER alpha in the context of a full length receptor, is required for synergy at multiple EREs. It appears that both the sub domains of AF-1: aa 41-64 and 87-108 are required for synergy when AF-2 is mutated. We propose to examine whether tamoxifen can mediate synergy under similar conditions. Since tamoxifen agonism appears to be promoter dependent, various promoter contexts will be utilized to compare the effects of estradiol and tamoxifen.

INTRODUCTION

Estrogens play an important role in the etiology of breast cancer (1,2). The hormone action is mediated by the two receptors ER alpha and beta expressed in the nucleus of target cells (3). Estrogen receptor is a hormone -inducible transcription factor, which binds to a *cis* acting element called estrogen responsive element, ERE. Most highly estrogen responsive genes contain multiple copies of estrogen responsive elements, EREs, that activate gene expression synergistically. Synergy is the observation when gene induction by two or more EREs is greater than the sum of induction by individual EREs when present alone. Although a number of models have been proposed for ER actions, the exact mechanisms of how estrogen receptor bound to multiple EREs cooperate to induce a synergistic response to estradiol, remains to be defined. Whether these mechanisms of synergistic up regulation of gene expression by estradiol, also apply to antiestrogens that have mixed agonistic and antagonistic prooperties, for *e g.* tamoxifen, is not understood. Analyzing the parameters that affect the extent of ER mediated synergistic response to estradiol and identifying region(s) in the receptor that mediate these actions have been the goals of this project. Knowledge obtained with ER alpha and estradiol, will be applied to understanding the mechanism of actions of ER beta and antiestrogens.

ANNUAL SUMMARY

Full length ER alpha cDNA (obtained from the laboratory of Dr. Angelo Notides, University of Rochester) was subcloned into pBluescript plasmid and various deletion mutants lacking ER alpha structural domains A, B, E and F were generated by PCR. The mutants were transcribed and translated *in vitro* to analyze their abilities to bind DNA and hormone. The full length and variant receptors were inserted into pM2AH, mammalian expression vector, kindly provided by Dr. Irving Boime, Washington University, St. Louis, MO. Three different ER negative cell lines, CHO, COS-1 and HeLa, were tested for the extent of ER mediated synergistic response to estradiol. This was performed by measuring the luciferase activities of reporters containing no, one, two or three EREs upstream of a minimal TATA box promoter. Since the highest extent of synergy was observed in COS-1 cells, this was chosen as a model to test other mutant receptors.

We found that A and F domains of ER alpha were not essential for synergy, however, the entire A/B domain was required. The receptor was transcriptionally inactive in the absence of E and F domains, as previously shown by others. The ability of the N-terminal AF-1 located in the A/B region to mediate synergy was measured, using a TAF2mut construct (gift of Dr. Donald McDonnell, Duke University) that contained a three amino acid mutation that destroyed the AF-2 function. The TAF2mut was capable of inducing synergistic response only in the presence of estradiol, suggesting that AF-1 is sufficient to mediate the synergy of multiple EREs at a TATA promoter, and this synergy is hormone-dependent.

We characterized the two sub domains, Box1 (aa 41-64) and Box2 (aa 87-108) of AF-1 in the context of functional and mutated AF-2. When AF-2 is functional, either Box1 or Box2 is sufficient for synergy, with Box1 being more important than Box2. When AF-2 is mutated, Box1 is absolutely essential for transcriptional activation, and both Box1 and Box2 are required for synergy.

We have preliminary evidence that AF-1 mediated synergy is cell and promoter specific. We will perform experiments to determine the requirements for estradiol-mediated synergy at different cellular and promoter contexts. We will subsequently use ER alpha in the presence of tamoxifen to compare the properties of estradiol and the agonistic effects of tamoxifen. These studies will then be extended to the recently discovered ER beta in the presence of estradiol and tamoxifen.

APPENDIX

Research accomplishments

- Construction of ER alpha variants
- Test of ER alpha wild type and variants for synergy at multiple EREs in COS-1 cells
- Identification of AF-1 as a dominant domain for ER-mediated synergy at a TATA promoter in COS-1 cells

Abstracts

- June '99 ENDO'99, The Endocrine Socitey's 81st annual meeting, San Diego, California. "Estrogen receptor alpha (ER alpha) amino-terminal activation function (AF-1) is required for estrogen-induced synergistic response at multiple EREs" **Sathya G**, Muyan M, Hilf R, Bambara RA. (poster presentation)

- September'98 Third annual scientific symposium, Univeristy of Rochester, Cancer center. "The Role of estrogen receptor (ER) N- and C-terminal activation domains on ER-mediated synergistic response at multiple ERE sites" **G. Sathya**, Muyan M, Driscoll MD, Bambara RA and Hilf R. (poster presentation)

Manuscripts

Role of amino-terminal activation function (AF-1) of estrogen receptor alpha (ER alpha) in estrogen-induced synergistic response at multiple EREs

G. Sathya, Mesut Muyan, Ping Yi, Robert A. Bambara and Russell Hilf* (*to be submitted*)

Abstract

Most highly estrogen responsive genes are synergistically activated by multiple copies of estrogen responsive elements (EREs) capable of binding estrogen receptor, ER. Properties of estrogen-induced synergistic response have been determined by several research groups including ours. The extent of synergy appears to depend on the number of EREs, their spatial separation and stereo-alignment with respect to the promoter. The exact events leading to synergistic induction of ER-mediated transcription remains to be defined. The classical ER alpha displays distinct domains for DNA binding, ligand binding and transcriptional activation. The two transactivation functions AF-1 and AF-2, located at the N- and C-terminus of the protein, respectively, can independently and cooperatively regulate gene expression in a cell and promoter specific manner. Guided by known ER alpha structure-function, we examined whether there is a domain in ER that mediates protein-protein interactions that promote synergy at multiple EREs. Using full length and truncated variants of ER alpha, we show in COS-1 cells that estrogen-induced synergistic response at a minimal TATA promoter requires the AF-1 function located in the amino terminus of ER alpha. AF-1 mediated synergy can be assayed only in the context of a full length receptor, containing a three amino acid mutation that destroys AF-2 function. Partial characterization of AF-1 revealed a dominant role for amino acids 41-64, which are required for AF-1 activity in the context of the AF-2 mutation. When AF-2 is mutated, both sub-domains of AF-1: amino acids 41-64 and 87-108 are essential for a synergistic response to estradiol. Our data support the idea that a hormone-induced conformational change and the overall structure of the full length receptor is required to activate AF-1. The presence of functional AF-1 and AF-2 is necessary to restore the full extent of synergy observed in the wild type receptor.

Introduction

Physiological effects of estrogens and other steroid hormones are mediated by their cognate receptors present in target organs (4). The classical estrogen receptor alpha (ER alpha) is a member of the steroid/nuclear receptor superfamily, which has a conserved structural and functional organization (5,6). ER alpha has six distinct functional domains including an activation function AF-1 at the N- terminus A/B domain, a DNA binding domain C (7), and a second activation function AF-2 within the C-terminal hormone binding domain E (8,9) (Figure 1). The DNA sequence to which the hormone-activated ER binds is called the estrogen response element, ERE (10,11).

A characteristic feature of estrogen responsive elements is that they are usually present in multiple copies in the regulatory regions of responsive genes (12). It is well established that multiple EREs synergistically activate estrogen response (13-18). Estrogen response from two or more EREs when greater than the added sum of response from individual EREs, is referred to as synergistic. The degree of synergy at multiple EREs depends on a variety of factors. Previous work from several laboratories, including ours, has shown that it depends on the number of EREs, their spacing and distance from the promoter (13,14,16,17,19).

Ptashne has proposed two models to explain synergism among receptor superfamily members (reviewed in (6)). In the first model, receptor dimers bind to two or more response elements in a cooperative fashion where the binding of one dimer facilitates the binding of a second dimer, resulting in greater occupancy of the response elements. Protein-protein interaction between the dimers would then stabilize the binding of adjacent receptor molecules leading to a synergistic up regulation of transcription. Although this model holds well for PR and GR, cooperative binding for ER has remained controversial (13,14,20-23).

In an alternative mechanism, protein-protein interactions between receptors bound to DNA and other target cofactors, which constitute the transcriptional machinery, result in synergistic enhancement of transcription. This model seems attractive not only for ER bound to multiple adjacent EREs, but also universally for activators that assemble at their specific sites on a promoter forming a nucleoprotein complex called the "enhanceosome" (24). In a recent review, Carey described a model where the enhanceosome, comprised of multiple activators bound to their cognate sites, displays a surface that is chemically and spatially complementary to a "target" surface present on coactivators and the basal transcriptional complex (24). It is conceivable that the complex of estradiol-liganded ER bound to multiple ERE sites has a surface designed to interact with coactivators and transcription machinery. This interaction produces the synergistic response to estradiol. This hypothesis predicts that we can identify a region in the receptor which when deleted causes the response to estradiol to be additive rather than synergistic.

It is well documented that the N-terminal activation function AF-1 and the ligand dependent C-terminal AF-2, can independently and cooperatively act to enhance transcription in a cell and promoter specific manner (8,25,26). Ligand-induced conformational change allows the association between the N- and C- terminus of the receptor, leading to interaction between AF-1 and AF-2 at most promoter sites (8,25,27,28). Previous studies on ER-mediated synergistic activation were performed in a variety of cell lines using N- or C-terminal truncation mutants of ER containing only AF-1 or AF-2 (8,17). These mutants not only exhibited cell- specific differences in their activation levels, but also differed in their ability to synergistically activate estrogen response, depending on the presence of other cis -acting elements in complex promoter regions (8,17,29). A recent report demonstrated the ability of AF-1 to function only in

the context of a full length ER (25). This led us to re examine the mechanism of ER-mediated synergy in a well defined and simplified system consisting of only ER-binding sites and the TATA box and using both truncated and full length ER .

We generated mutant forms of ER alpha that have an intact DNA binding domain and nuclear localization signals but lack one or more of the other structural and functional domains. Using an ER negative COS-1 cell line, we assayed the level of synergy mediated by wild type and variant ERs, by measuring the activity of a luciferase reporter regulated by up to three EREs upstream of a minimal TATA box promoter. Our results suggest that the overall structure of the receptor, including the conformational change introduced by estradiol, affects the extent of synergistic activation mediated by the receptor. In the context of a full-length receptor, AF-1 appears to play a dominant role in mediating activation as well as synergy. We determine the role of specific amino acids in AF-1 essential for this activity.

Materials And Methods

Plasmids and expression vectors

ER alpha cDNA (a gift of Dr. Notides, University of Rochester) was subcloned into the *Eco*RI site of pBlueScript KS vector (Stratgene) and subsequently inserted between the *Sal* I and *Bam* HI sites of the mammalian expression vector pM2AH (gift of Dr. Irving Boime, Washington University, St.Louis, MO), under the regulation of HaMuSV-LTR promoter (Muyan *et al.*, 1999) (Figure 1). ER alpha truncation mutants were generated by PCR with the introduction of *Sal* I and *Bam* HI sites at the 5' and 3' ends and inserted into pBluescript vector. Kozak consensus sequences were placed at the 5' end of each construct to ensure efficient translation. ΔC, ΔBox1, and ΔBox2 were generated by deleting sections of the cDNA corresponding to the indicated amino acids using appropriately designed PCR primers. TAF2mut was generated by reinserting into wild type (Figure1), a PCR amplified fragment from ER-TAF1 (kindly provided by Dr. Donald McDonnell, Duke University, NC) that contains a three amino acid modification destroying AF-2 function of ER alpha (25). TAF2mut Δ Box1 and TAF2mut ΔBox2 were constructed by inserting *Sal* I- *Fse*I fragments of ΔBox1 and ΔBox2 into TAF2mut. All constructs with the exception of BCDEF had a hexahistidine-tag at the C-terminal to facilitate protein purification, if necessary. The presence of histidine tag did not affect the DNA binding and transcriptional properties of the wild type ER (data not shown) and therefore placed at the C-terminus of most of the constructs. The cDNA sequences in all constructs were verified (Core nucleic acid facility, University of Rochester) and subsequently inserted into pM2AH mammalian expression vector using *Sal* I and *Bam* HI restriction sites.

Luciferase reporter constructs used in this study were generated by inserting the *Hind* III/*Nco* I fragments containing 1, 2, or 3 tandem EREs upstream of the vitellogenin B1 TATA box, from CAT reporter vectors (16) into pGL3-Basic luciferase reporter (Promega). The TATA-luc reporter was constructed by removing the three EREs from 3ERE-TATA construct by *Xho* I digestion and religating the vector DNA. All plasmids were purified using Qiagen plasmid maxi prep for transfection studies.

In vitro transcription and translation and Gel mobility shift assays

In vitro transcription and translation reaction was performed from supercoiled pBlueScript constructs using TNT reticulocyte lysate system and T3 polymerase (Promega) as per manufacturers' instruction with S^{35} Methionine (Dupont NEN). Translated protein sizes were verified on a 10% SDS-PAGE. Proteins transcribed and translated under non-radioactive conditions were run on a 10% SDS-PAGE and detected using ER specific antibodies (HC-20 to ER alpha F domain from Santa Cruz

Biotechnology Inc. or EVG-F9 to A/B domain, a gift from Abdul M. Traish, Boston University) with ECL+PLUS detection system (Amersham), and used in gel shift analyses .

A double stranded oligomer containing a single copy of the 17 bp perfect consensus ERE of the chicken vitellogenin II gene was obtained by annealing equimolar amounts of single stranded oligomers synthesized and PAGE purified at Genosys Biotechnology Inc (The Woodlands,TX), as previously described (30). The double stranded ERE was 3' end labeled using $\alpha[^{32}P]$ dTTP and 5'-3' exo (-) Klenow fragment (New England Biolabs) according to the manufacturers' instructions. Approximately 60,000 cpm of labeled ERE were mixed with 5-10 µl of *in vitro* transcribed and translated protein, 1.5 µg of poly dI-dC (Midland Certified reagents), with or without antiserum (1 µl Anti-his antibody (Qiagen Inc.) or 1 µl 1:10 diluted H222 (Abbott laboratories), in a total volume of 50 µl in TDPEK 100 (40 mM Tris HCl pH 7.5, 1 mM DTT, 0.5 mM PMSF, 1 mM EDTA, 100 mM KCl) +0.1% NP-40, +20% glycerol and incubated on ice for one hour. 40 µl of each reaction were loaded on a 5% native polyacrylamide gel and electrophoresed at 150V for 1.5 hours as described previously (30). The gel was dried and exposed to X-ray film at -80°C.

Hormone Binding assays

Aliquots of 2 µl of the translation mixtures were incubated overnight with 100 pM I^{125}-17β-estradiol, with or without 300 -fold excess non-radioactive estradiol, in a hormone binding buffer containing 20 mM HEPES, pH 7.9, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 6mM monothioglycerol and 10 mM sodium molybdate at 4°C (31) in a final reaction volume of 200 µl. Bound and free ligands were separated using BioRad G25 spin columns and bound radioactivity was measured using scintillation counting.

Cell culture, transfections, Western Blotting and reporter assays

COS-1, African green monkey kidney cells, obtained from ATCC (Rockville, MD), were maintained in DME high glucose medium (Tissue culture support center, University of Washington, St. Louis) with 10% Fetal Bovine serum (Hyclone) and 0.5 % Penicillin and Streptomycin (Life Technologies, Inc.).

Approximately $3x10^5$ cells were plated overnight in each well of a 6-well tissue culture plate. Two µg of the mammalian expression vector pM2AH either expressing no protein or ER alpha wild type or variants, were transfected into each well using 10 µl superfect transfection reagent (Qiagen). At 36 hours after transfection, the cells were lysed in 200 µl RIPA buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 0.5 mM PMSF and 1 µg/ml (leupeptin/ aprotinin) protease inhibitors. The protein concentrations were estimated by BioRad protein assay and 30 µg of total protein were loaded on a 10% SDS-PAGE. The gel was immunoblotted with EVG-F9 or HC 20 antibodies using ECL +PLUS detection system (Amersham)

For transactivation assays, $2x10^5$ cells were cultured overnight in each well of a 12-well plate in phenol red-free medium supplemented with 5% charcoal-dextran stripped FBS (Hyclone) and antibiotics. A total of 1 µg DNA containing 0.2 µg of pCMV β-galactosidase internal control plasmid, 0.5 µg of luciferase reporter constructs containing no, one, two or three EREs upstream of a minimal TATA box promoter and 0.3 µg of pM2AH containing ER wild type or variant cDNA was transfected per well using 5 µl of superfect transfection reagent (Qiagen) as per manufacturers' directions. After incubation for three hours at 37°C, the cells were washed once with 1X PBS (Phosphate Buffered Saline, pH 7.4) and treated with or without 1 nM estradiol diluted in phenol red-free medium containing 1% stripped FBS and antibiotics. After 24 hours, the

cells were lysed in 200 µl 1X Reporter lysis buffer (Promega) to perform luciferase and β-galactosidase assays as described previously (16). The luciferase activity was normalized by β-galactosidase activity and the relative luciferase activity was measured by setting the value for TATA-luc construct in the absence of estradiol to 1. Each experiment was performed at least three times in duplicate.

Statistical analysis

Using the normalized luciferase values in the presence of estradiol for each receptor type (wild type or variant ER alpha), a t-test was used to test the null hypothesis that the mean for the 2ERE construct was twice the mean for the 1ERE construct, against the one-sided alternative that it was greater than twice the mean for the 1ERE construct. Similar one-sided t-tests were used to test whether the mean for the 3ERE-construct was greater than 1.5 times the mean for the 2ERE construct, or that it was greater than 3 times the mean for the 1ERE construct. A single, pooled variance estimate was used for all tests for a given receptor type, obtained by fitting a one-way analysis of variance model to the log transformed data for that receptor type.

Immunocytochemistry

COS-1 cells were plated on sterile cover slips placed into the wells of a 12-well plate and transfected with 1 µg of vector expressing ER alpha wild type or variant. After 24 hours, the cells were washed three times in 1X PBS and fixed with 2% paraformaldehyde for 30 minutes and permeabilized with 0.4% Triton X-100 in 1X PBS for 10 minutes. After three washes with 1X PBS, the cells were blocked in 10% normal goat serum for an hour. Cells were incubated for an hour in 1:100 dilution of primary antibody in 2% normal goat serum (HC20 or EVG-F9 antibodies were used). After three washes with 1X PBS, 1:200 dilution of fluorescein-conjugated secondary antibody (Santa Cruz Biotechnology) was added to the cells and incubated for 30 minutes. After washing with PBS, the cover slips were mounted on glass slides using mounting solution containing DAPI (Vectashield).

Results

Wild Type ER alpha and variants bind ERE in vitro

Figure 1 shows the schematic of ER alpha derivatives used in this study. ER alpha was systematically truncated to remove individual domains A, B, C, E and F. The D region was retained in all variants since it has the nuclear localization signal (32). The entire A/B or EF regions were removed to generate variants that had only AF-1 (ABCD) or AF-2 (CDEF) activity. ER alpha AF-1 activity has been characterized to contain two sub domains Box1 (amino acids 41-64) and Box2 (amino acids 87-108) (33). ΔBox1 and ΔBox2 variants had the amino acids corresponding to these two sub-domains of AF-1 removed (see Materials and Methods). TAF2mut is a full length ER with a three amino acid mutation that destroys the AF2 activity but retains the wild type DNA binding and hormone binding activities (25) (see Materials and methods). TAF2mutΔBox1 and TAF2mutΔBox2 lack either Box1 or Box2 in the context of the AF-2 mutation. The wild type and variants were transcribed and translated *in vitro* and used in DNA binding assays with an oligomer containing the 17 bp consensus ERE sequence. In Figure 2, lanes 1-12 contained the vector control, wild type receptor, ΔBox1, ΔBox2, TAF2mut ΔBox1 and TAF2mut ΔBox2 incubated with ^{32}P labeled ERE with (lanes 2, 4, 6, 8, 10, 12) or without (lanes 1, 3, 5, 7, 9 and 11) anti-His antibody to supershift the bound ERE complexes. Lanes 13 -24 contained the TAF2mut, BCDEF, CDEF, ABCDE, ABCD and ΔC incubated with (14, 16, 18, 20, 22 and 24) or without (13, 15, 17, 19, 21 and 23) the supershifting antibody. There is a band (labeled NS) appearing in all the lanes including the vector lane. This is a protein in the lysate that binds non-specifically to the unannealed single strand of the labeled ERE.

The ΔC (lanes 23 and 24) produced neither a shifted complex with the ERE nor a supershift with the antibody, as expected. All the other variants bound the ERE and were supershifted by the antibody. Interestingly, the binding of ABCD to the labeled ERE was weak (lane 21), however, the addition of antibody intensified the supershift (lane 22). This observation was reported earlier by others who attributed the weak binding of ABCD to ERE to the lack of dimerization domain located in the E region (34). The antibody is thought to somehow stabilize weak interaction between the protein and DNA producing a strong supershifted band. This interpretation was offered for another ER alpha antibody by Fawell *et al* (35). As shown below and already shown by other laboratories (25), the absence of the E and F region in this construct also reduces its activation capability in transfection assays. We also tested the hormone binding ability of wild type and variant ERs transcribed and translated *in vitro,* and detected specific binding of I^{125} estradiol to all constructs containing the E domain (data not shown).

ER wild type and variants are expressed and detected in COS-1 cell nuclei

COS-1 cells were transfected with the mammalian expression vector either expressing no cDNA, wild type or variant ER alpha cDNA and the extracts were used for Western blot analysis of the protein. In Figure 3, lanes 1-4 were probed with antibody to the A/B region and 5-15 were probed using antibody to the F region. The antibodies detected approximately equal levels of the wild type receptor and variants, suggesting similar levels of expression of the constructs in COS-1 cells. Using staining *in situ* with fluorescein-conjugated antibody to detect the protein and DAPI to detect cell nuclei (Figure 4), we observed the nuclear localization of all variant and wild type receptors. The control EF shown in Figure 4 was derived from cells transfected with vector expressing only 'EF' region of ER alpha, localizing to the cytoplasm.

Synergistic response to estradiol occurs in the absence of A or F domains of ER alpha

We first examined whether ER alpha wild type can cause a synergistic increase in reporter activity with increasing number of EREs in COS-1 cells. Luciferase constructs containing no, one, two or three EREs were transfected along with the expression vector expressing wild type ER alpha. Luciferase activities in the absence and presence of estradiol from each reporter was normalized relative to the basal activity from TATA-luciferase plasmid in the absence of estradiol set to 1. As shown in Figure 5, the relative luciferase activities induced by estradiol in the presence of wild type ER were 1.3, 18.4 and 120.6 in the presence of 1, 2 or 3 EREs, respectively. Statistical analysis showed that the response was highly synergistic in the presence of two and three EREs. In the absence of exogenous estradiol, the values were 0.9, 1.3 and 2.4, respectively, suggestive of a modest increase in the absence of hormone. The pattern of response in the presence of estradiol for BCDEF and ABCDE variants was similar to that of the wild type, showing synergy in the presence of two and three EREs. Interestingly, transfection of the BCDEF construct showed larger increase in luciferase activities compared to the wild type, in the absence of exogenous estradiol. The relative luciferase values were 1.0, 2.1 and 10.4 for one, two and three EREs, respectively. These values increased more steeply with increasing number of EREs than those of the wild type receptor. This could be due to increased ligand-independent activity (36) or increased sensitivity of the BCDEF variant to trace amounts of estradiol present in the growth media, shown previously to affect ER alpha transcriptional activity (37). Nevertheless, in the presence of estradiol, the pattern and levels of reporter activity for BCDEF are very similar to that of wild type receptor, suggesting that the N-terminal A region does not contribute to the estradiol-induced synergistic response. Although ABCDE induced a synergistic pattern for the luciferase activity in the presence of estradiol, the amount of activity for the three EREs was about 50% of that of the wild type. Although the F region is not essential, it appears to play a role in augmenting the level of synergy in the wild type receptor.

ER alpha AF-1 is required for estrogen- induced synergistic response

Since deletion of A region did not affect the pattern of estradiol-induced luciferase activity in the presence of multiple EREs, we created a truncation mutant that lacked the entire A/B region. In the CDEF construct, AF-1 function was removed leaving only AF-2 to mediate transcription activation. Luciferase activity mediated by CDEF in the presence and absence of estradiol is shown in Figure 6. Enhancement of luciferase activity was observed only in the presence of estradiol, as was expected. The activities were 1.2, 1.7 and 3.5 for one, two and three EREs, respectively, in the presence of estradiol. Statistical analyses indicated that these numbers display only an additive increase in response. Increasing the amount of transfected CDEF expression plasmid did not change the pattern of response shown here (data not shown). We therefore concluded that the A/B region or AF-1 of ER alpha is essential for mediating synergy at multiple EREs in the presence of estradiol.

AF-1 mediated response to estradiol is synergistic in the context of a full length ER

From the experiment above we learned that AF-1 is required for mediating estradiol-induced synergy. To test whether AF-1 can mediate synergistic response in the absence of AF-2, we created the ABCD mutant lacking both E and F domains. This removed the hormone binding and AF-2 activity of the receptor. In transfection assays, the mutant caused no increase in luciferase activity even in the presence of three tandem EREs (Figure 7). This outcome was not surprising since earlier reports also demonstrated the lack of transcriptional activity in ABCD (25,38). It was attributed to the lack of dimerization resulting from the absence of E domain (34,35). To avoid a dimerization problem, we tested the synergistic ability of AF-1 using the TAF2mut construct which contains amino acid changes at positions 538, 542 and 545 that destroy AF-2 activity in the full length receptor (25). Interestingly, as shown in Figure 7 and as demonstrated previously (25), luciferase activity was enhanced by TAF2mut only in the presence of estradiol. This suggests that ligand was required to mediate AF-1 dependent activation. The luciferase activities in the presence of one, two and three EREs were 1.0, 2.9 and 10.9, respectively. Statistical analyses showed significant synergy for three EREs but not for two, indicating that AF-1 can mediate synergistic response to estradiol but to a lesser extent than the wild type, which synergizes with two EREs. It also suggests that both AF-1 and AF-2 are required to restore full receptor activity, in turn increasing the level of synergistic response in the presence of multiple EREs.

AF-2 mutated receptor requires both Box1 and Box2 sub-domains of AF-1 to mediate synergy

Since deletion of A/B region resulted in additive response to estradiol (Figure 6), we tested the deletions of smaller sections within the A/B region previously characterized as part of the AF-1 activity. In a recent report, McInerney *et al* (33) described two such regions termed Box1 and Box 2, corresponding to amino acids 41-64 and 87-108, respectively. Box1 was found to be responsible for antiestrogen agonism and Box2 was suggested to play a role in estradiol-stimulated transcriptional activity. We recreated these deletions in the wild type receptor and used them to test the role of these regions in ER-mediated synergistic response. The luciferase activities in the presence of estradiol for one, two and three EREs were 1.1, 4.3 and 14.8, respectively, for the Box1 deletion and 1.0, 7.4 and 37.5, respectively, for the Box2 deletion (Figure 8). Both sets of numbers represent statistically significant synergy, with trends similar to the wild type receptor. We therefore conclude that either Box1 or Box2 is sufficient for ER mediated synergistic response to estradiol, in the context of a functional AF-2.

It is worth noting however, that Box1 or Box2 deletions lowered the overall activity of the reporter compared to the wild type receptor. The wild type ER estradiol-

induced luciferase activities were 1.3, 18.4 and 120.6. The Box1 deletion also clearly had a greater effect than the Box2 deletion. In contrast, McInerney *et al* observed no change in estradiol-induced transcription activity for the Box1 deletion mutant (33). One possible explanation lies in the differences in cell types (HEC-1 and MDA-MB-231) and promoters (pS2 gene promoter) used in their study *versus* ours. In their paper, the authors also reported the findings of Metzger *et al* (39) in CEF cells where the deletion of aa 51-149 abolished the transcriptional activity of human ER on ERE-TATA CAT and aa 51-93 and aa 102-149 independently synergized with AF-2. It therefore appears that the location of AF-1 activity varies considerably depending on the cell and promoter contexts. In our assays performed in COS-1 cells, the Box1 sub region appears to be more critical than Box2 for estradiol-induced synergy, at a minimal TATA promoter. To confirm this, we deleted the Box1 or Box2 sub-domains in the context of the AF-2 mutation. As shown in Figure 8, the Box1 deletion plus AF-2 mutation results in a complete loss of activity, suggesting a dominant role for the Box1 sub-domain. When Box2 was deleted, transcriptional activity was still evident from increase in luciferase activity in the presence of estradiol. However, this increase in activity did not follow a synergistic pattern. This suggests to us that while Box1 is contributing to the AF-1 function, it is not sufficient to induce synergistic response to estradiol. Both Box1 and Box2 are required to cause a synergistic response when AF-2 is mutated.

Discussion

Synergism is an important factor in the biology of estrogen action. Transient fluctuations in hormonal levels are sensed by target organs which respond to the hormone by altering local gene expression patterns (40,41). Gene transcription in cells occurs in a milieu of proteins that include activators that bind to cis- acting elements, coactivators, the general transcriptional machinery and the components of the chromatin within which the gene resides (42). The multi- protein complex at a promoter is in itself undergoing interactions that synergistically modulate gene expression patterns in a gene- and cell-specific manner. Synergistic enhancement of transcription has been reported for several diverse eukaryotic transcriptional activators including GAL4, GCN4, glucocorticoid receptor, and between estrogen and progesterone receptors (21,24,38,43-49).

ER mediated synergistic activation at multiple EREs has been investigated by several laboratories using perfect consensus EREs or imperfect EREs of the vitellogenin B1 gene(8,13,14,16-18,22,29). Synergistic interactions between ER and other upstream activators has also been reported (29,49). It was reported that ER truncation mutants, containing only AF-2 and not AF-1, can independently synergize to activate reporter gene expression in response to estradiol (8,17). It appeared from those reports that synergism at multiple ERE sites does not require AF-1 interaction with AF-2. However, it was recently reported that the AF-1 function of human ER is highly specific to cell and promoter contexts and is active only in the context of a full length ER (25). Also, truncated Xenopus ER lacking the N-terminal AF-1 was shown to synergize with upstream activators, even better than the wild type XER (29), implying that presence of nearby cis -acting elements affect the synergistic properties of truncated ER. Therefore, use of both full length and truncated ERs at a minimal TATA box promoter was deemed necessary to address the specific roles of ER alpha functional domains in ER-mediated synergistic response at multiple ERE sites.

Here, we detail efforts to dissect the estrogen receptor, in order to identify a region that is directly responsible for the synergy function in a gene regulated by multiple tandem EREs. To eliminate possible interactions between ER and other activators that bind to complex promoters, we used a minimal TATA box promoter regulated by single or multiple EREs. We tested several ER negative cell lines –HeLa, CHO and COS-1- for

relative levels of luciferase activation mediated by wild type ER and estradiol. The relative activities for one, two and three EREs in the presence of estradiol were 1.3, 10.9 and 19.2, respectively, in HeLa cells, and 0.3, 5.2 and 13.5, respectively, in CHO cells. The reason for an apparent suppression in luciferase activity in the presence of one ERE in CHO cells is unclear. Although synergy with wild type ER is evident in HeLa and CHO cells, the magnitude of both activation and synergy is much lower than in COS-1 cells where the luciferase activities were 1.3, 18.4 and 120.6, respectively, for the full length ER alpha. Therefore, we selected to study COS-1 cells, wherein one can unambiguously detect synergy for most of the variant receptors.

Our observations suggest that estradiol plays an important role in producing the synergy detected with the luciferase gene. Wild type ER alpha and all variants with the exception of BCDEF were capable of activating and/or inducing synergy *only* in the presence of estradiol. We found that even the "hormone-independent" AF-1 activity is evident only when exogenous estradiol is added. This suggests that ligand is required for receptor activation and synergy at multiple ERE sites. The role of ligand in ER transcriptional activation has been emphasized by others who concluded that there is no ligand -independent activity of the wild type ER in cultured cells (37,50). The lack of synergy in CDEF mutant indicates a requirement for the N-terminal region to restore the synergy function. Our measurement with the TAF2mut confirmed the earlier observation that AF-1 is functional only in the context of a full length receptor (25). With this mutant we were able to measure the hormone -dependent AF-1 activity, in the context of AF-2 mutation, and were able to demonstrate that AF-1 is sufficient to induce synergy in COS-1 cells. AF-1 is therefore not only important for ER transactivation (25) and repression by dominant negative mutants (51), but also for mediating synergistic interactions at multiple EREs, as shown by our studies. This also implies that this region of ER plays a key role in mediating protein-protein interactions. Partial characterization of AF-1 in COS-1 cells, using Box1 and Box2 deletions, suggests that synergy may be coupled to the activity of AF-1 itself. In the context of a mutated AF-2 function, we ascertained the role of AF-1 sub-domains Box1 and Box2. Our data show that Box1 mediates AF-1 activity, but is not sufficient to mediate synergy which requires both Box1 and Box2.

In contrast to our findings in COS-1 cells, it was reported that AF-2 can independently synergize in HeLa and chicken embryo fibroblast cells in the absence of AF-1 (8). In that study, the authors used chimeric proteins containing 'AB' or 'EF' domains of ER alpha fused to GAL4 DNA binding domain to test synergistic induction at tandem GAL4 binding sites upstream of the globin gene promoter. The use of a heterologous DNA binding domain and a complex promoter to test the synergy of AF-1 and AF-2, are likely reasons for the differences between their findings and ours. Also, more recently, Xing *et al* observed that the N-terminal deletion mutant of Xenopus ER (XER) lacking AF-1 activity was a weak transactivator, however, it was better than the wild type XER in synergizing with upstream activators, such as NF1, AP1 or vitellogenin activator upstream binding proteins (29). The authors suggest that the presence of upstream activators compensate for the lack of AF-1 activity in XER. This indicates that the ability of a truncated estrogen receptor to synergistically activate estrogen response at multiple EREs is influenced by the nature of nearby promoter elements, due to possible heterosynergistic interactions. This further supports our conclusion that AF-1 is required for homosynergism at multiple EREs.

Our observations in CHO and HeLa cells with TAF2mut construct differed from those we obtained with COS-1 cells. The estradiol-induced relative luciferase values with TAF2mut for one, two and three EREs were 0.2, 1.2 and 3.2, respectively, in CHO cells and 1.5, 2.2 and 4.2, respectively, in HeLa cells. These responses did not represent a synergistic pattern. This suggested that AF-1 is not sufficient to mediate synergy in

CHO and HeLa cells. CDEF induced estrogen response in HeLa and CHO cells was also additive (data not shown). This implies that at certain cellular contexts both AF-1 and AF-2 are required for a synergistic response to estradiol. As mentioned previously, ER wild type induced synergy in CHO and HeLa cells occurred to a much lower extent than in COS-1 cells, suggesting that the level of synergy exerted by ER depends on cellular factors other than ER. Varying levels of intermediary coregulators (52) that interact with AF-1 and AF-2 may contribute to differences in the level of synergistic response among cell types. This could also contribute to the lack of AF-1 mediated synergy in CHO and HeLa cells. The presence of both AF-1 and AF-2 significantly enhanced the extent of synergy in all cell lines tested. In the full length ER, it is possible that coactivators that interact with both AF-1 and AF-2 (*e.g* SRC-1) (53), may not only promote interactions between AF-1 and AF-2 within a single ER homodimer, but also between homodimers bound to multiple EREs, leading to synergistic activation of the target gene. The presence of multiple NR boxes in coactivators and their possible role in mediating synergy among nuclear receptors was discussed in a recent article (54). With the identification of specific coactivators that interact with ER AF-1 and/or AF-2 (52), we can now begin to address the roles of coactivators in ER-mediated synergy using a cell free system with purified full length and variant ERs and individual coactivator molecules in the context of simple and complex promoters.

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Figure Legends

1. Schematic of ER alpha constructs used in this study

Wild type ER alpha with the amino acids corresponding to structural domains A/B, C, D, E and F are shown. Derivatives BCDEF, CDEF, and ABCDE were derived by PCR amplification of the cDNA corresponding to appropriate amino acids. TAF2mut had the three amino acid mutation in the E domain that destroys AF-2 function. ΔC lacked the DNA binding domain C. ΔBox1 and ΔBox2 variants were obtained by deleting appropriate regions within AF-1 of A/B domain. TAF2mut ΔBox1 and TAF2mut ΔBox2 lacked Box1 or Box2 subdomain in the context of a mutated AF2 function.

2. DNA binding analyses using in vitro transcribed and translated proteins

pBluescript vector containing no cDNA, cDNA coding for the wild type or variant ER alpha were transcribed and translated *in vitro* and proteins were used in a binding reaction with ^{32}Plabeled 17 bp ERE as described in Materials and methods. The receptor type used in the binding assay is indicated on the top of the gel. -/+ indicate absence or presence of supershifting antibody. The position of free ERE, ER-ERE complex and Ab-ER-ERE supershifted complex are as indicated. The faster migrating bound ERE in lane 17 is indicated by an asterisk *. NS refers to a non-specific band appearing in all lanes including the vector translated lanes.

3. Expression of ER constructs in COS-1 cells

COS-1 cells were transfected with mammalian expression vector pM2AH expressing no CDNA, ER full length cDNA or cDNA corresponding to an ER variant. 30 μg of total protein was loaded into each lane of a 10% SDS-PAGE and immunoblotted using EVG-F9 (antibody to the A/B domain, lanes 1-4) or HC-20 (antibody to F domain, lanes 5-15). Molecular weight standards migrated as indicated to the left of each gel and protein sizes were as expected. NS refers to a non-specific band appearing in all lanes including 1 and 5, loaded with extracts from cells transfected with vector only.

4. ER wild type and variants localize to COS-1 cell nucleus

COS-1 cells grown on sterile coverslips were transfected with 1μg of expression vector expressing ER wild type or variant. After 24 hours, the cells were fixed to detect proteins using ER-specific antibodies and FITC-conjugated secondary antibodies using a fluorescent microscope. DAPI staining was used to identify nuclei. All ER variants used in the study localized to the nucleus. As a control, in the last panel cells were transfected with cDNA coding for only EF domain which localized exclusively to the cytoplasm.

5. Estradiol-induced synergistic response at multiple EREs does not require A or F domain of ER alpha

COS-1 cells were transfected with luciferase reporter containing no (TATA-luc), one (1ERE-TATA), two (2ERE-TATA) or three (3ERE-TATA) EREs along with vector expressing full length ER wild type (WT), variants BCDEF or ABCDE and pCMV βgal internal control and treated for 24 hours in the absence or presence of estradiol (1nM). The extracts were assayed for luciferase and β-galactosidase activities and the luciferase/β galactosidase activities was normalized by setting the ratio for TATA-luc in the absence of estradiol to 1. Shown in the graph are values of normalized activities in the presence of added estradiol obtained from a minimum of 3 experiments performed in duplicate.

6. ER alpha AF-1 is required for estrogen- induced synergistic response

COS-1 cells were transfected with luciferase reporter containing no (TATA-luc), one (1ERE-TATA), two (2ERE-TATA) or three (3ERE-TATA) EREs along with vector expressing CDEF variant and pCMV βgal internal control and treated for 24 hours in the absence or presence of estradiol (1nM). The extracts were assayed as described in legend to Figure 5. The relative luciferase activities in the absence and presence of estradiol from five independent experiments performed in duplicate are represented in the graph.

7. AF-1 mediated response to estradiol is synergistic in the context of a full length ER

COS-1 cells were transfected with luciferase reporter containing no (TATA-luc), one (1ERE-TATA), two (2ERE-TATA) or three (3ERE-TATA) EREs along with a vector expressing ABCD or TAF2mut variant and pCMV βgal internal control and treated for 24 hours in the absence or presence of estradiol (1nM). The extracts were assayed as described in legend to Figure 5. The relative luciferase activities in the absence and presence of estradiol from three independent experiments performed in duplicate are shown in graph.

8. ER alpha requires both Box1 and Box2 sub domains of AF-1 to mediate synergy when AF-2 is mutated

COS-1 cells were transfected with luciferase reporter containing no (TATA-luc), one (1ERE-TATA), two (2ERE-TATA) or three (3ERE-TATA) EREs along with a vector expressing ΔBox1 (Box1 del), ΔBox2 (Box2 del), TAF2mutΔBox1 (TAF2mut Box1del) or TAF2mutΔBox2 (TAF2mut Box2del) and pCMV βgal internal control and treated for 24 hours in the absence or presence of estradiol (1nM). The extracts were assayed as described in legend to Figure 5. The relative luciferase activities in the presence of estradiol from three independent experiments performed in duplicate are shown in graph.

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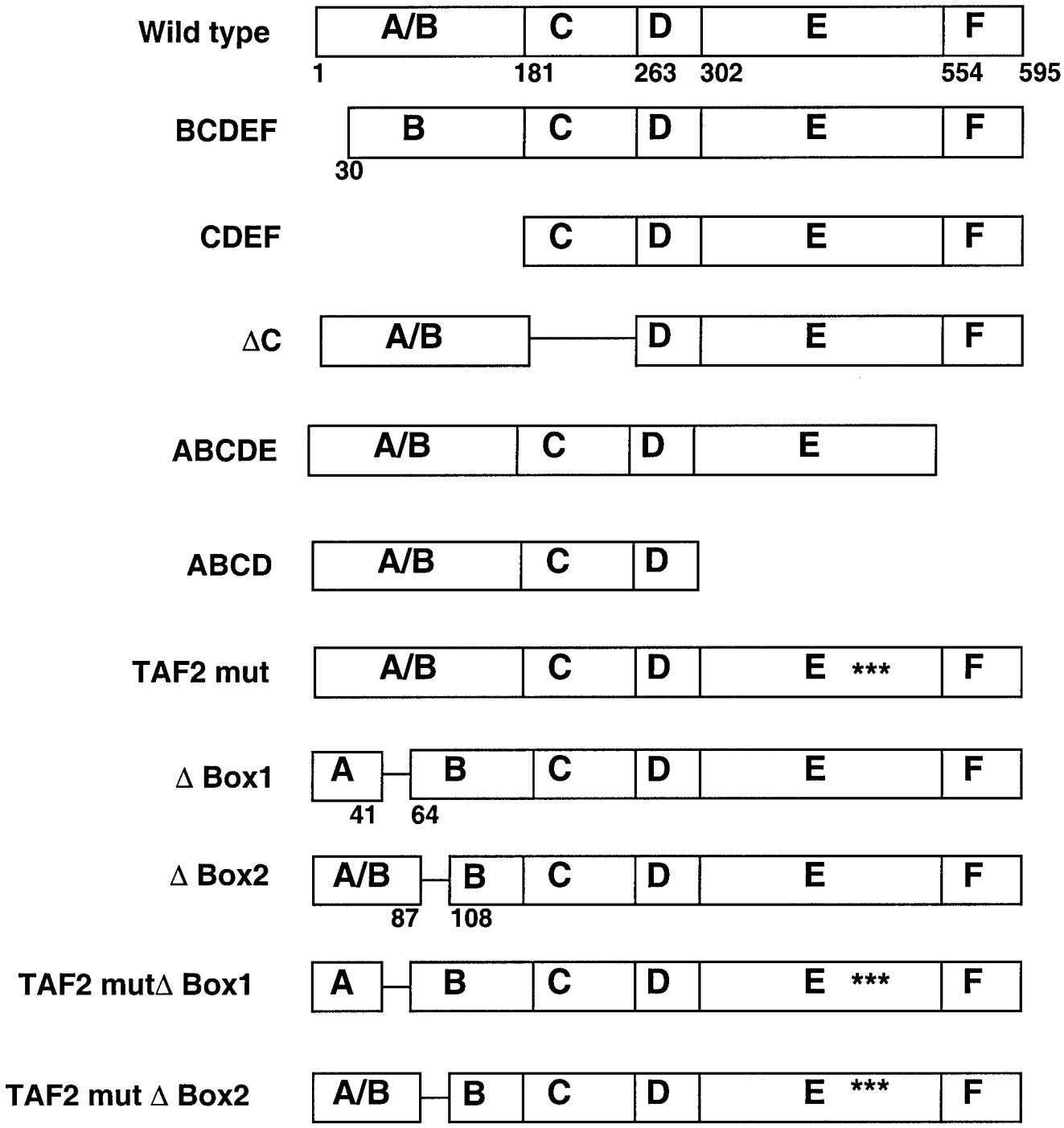
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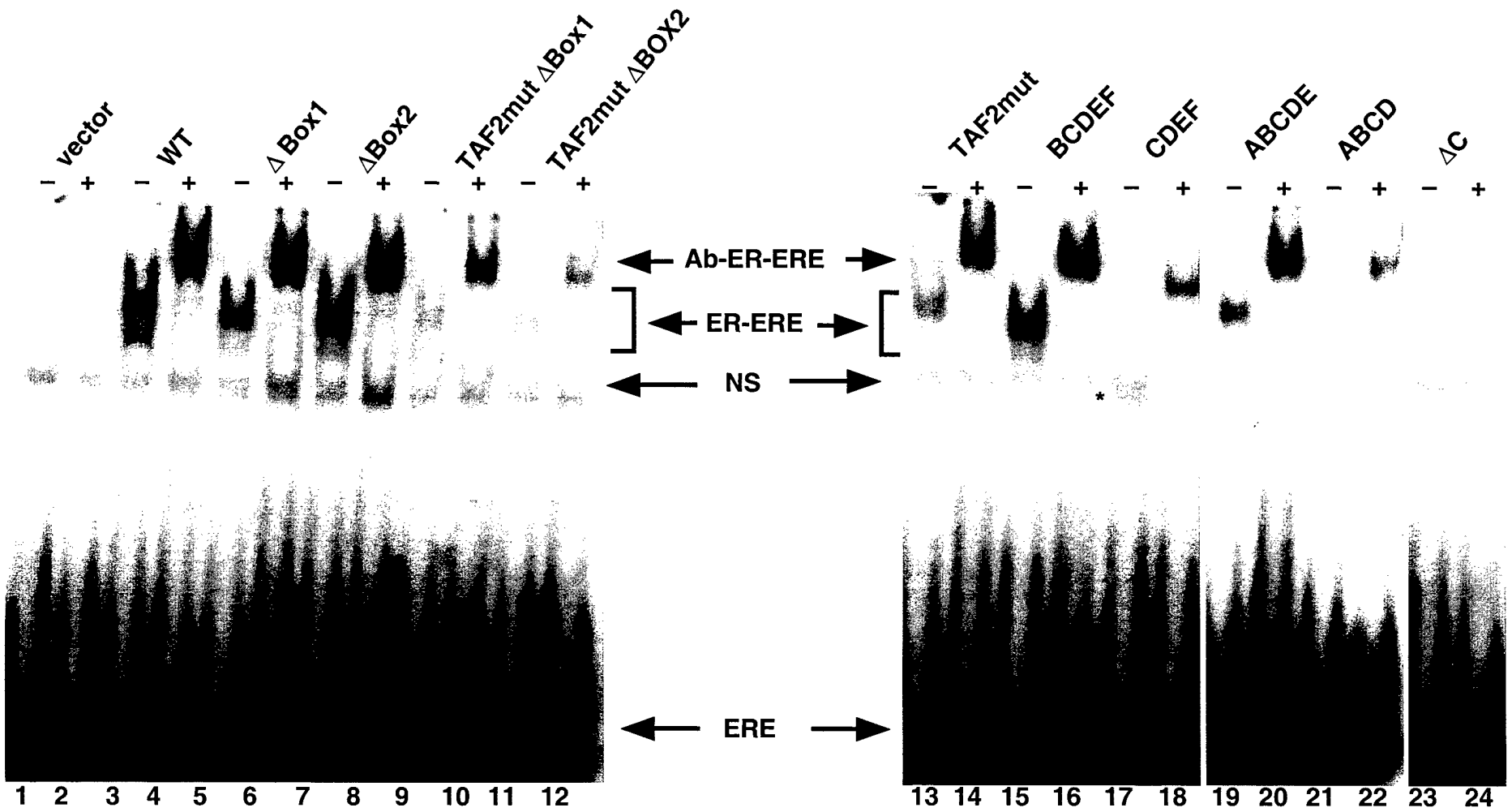
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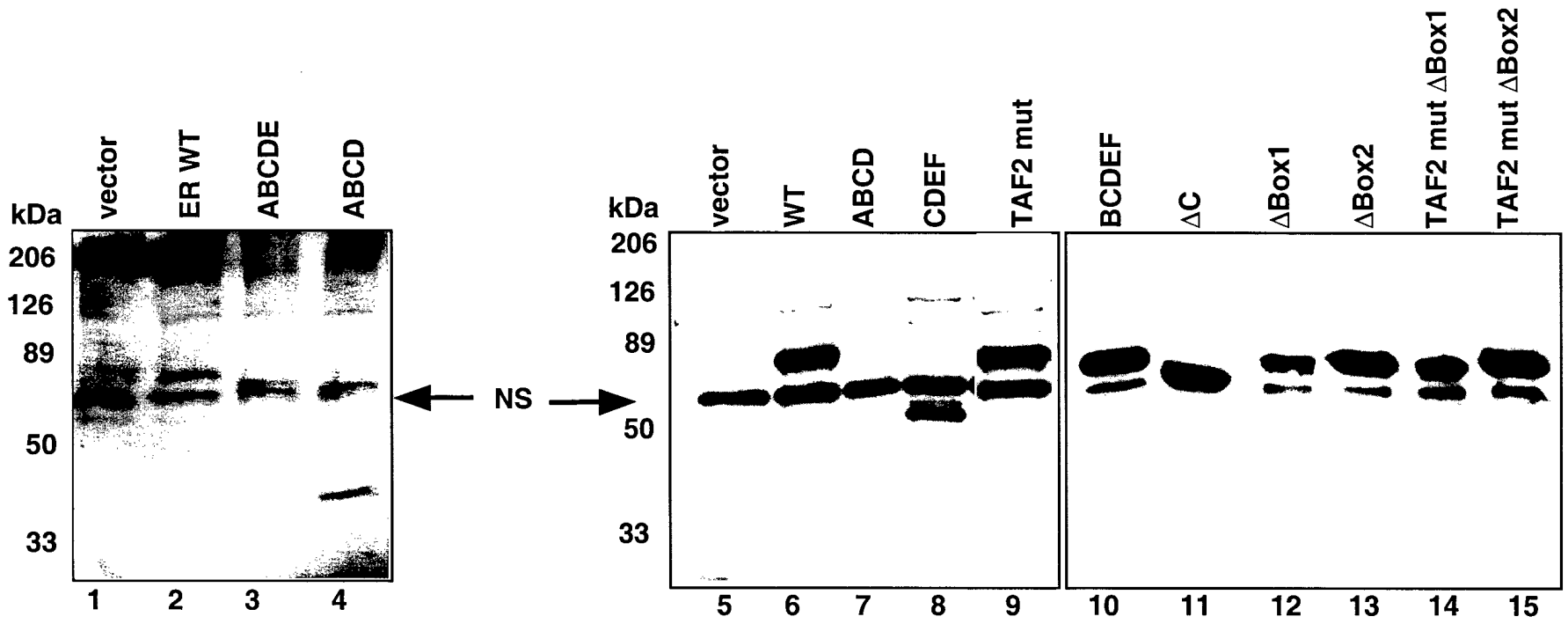
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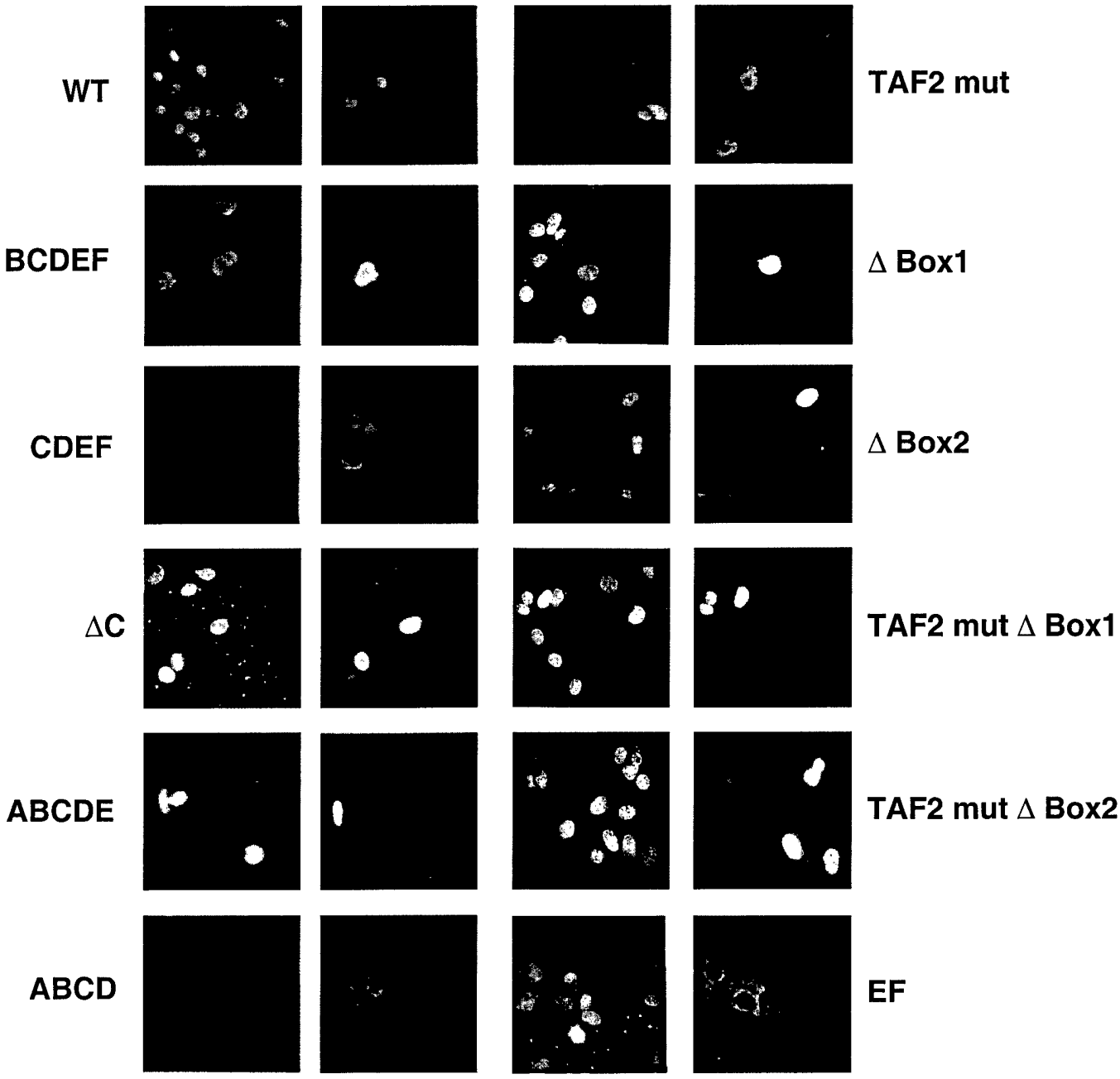
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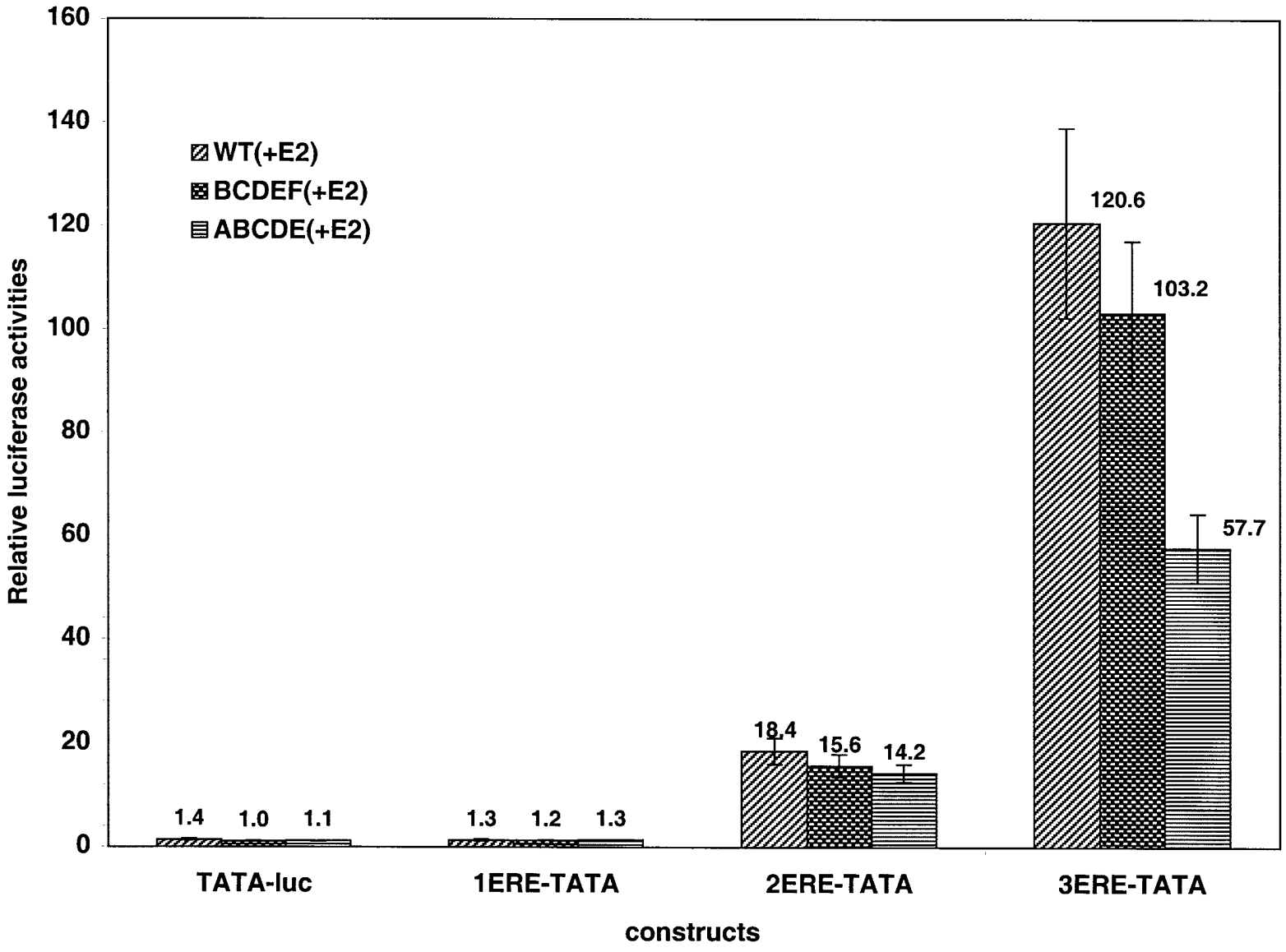
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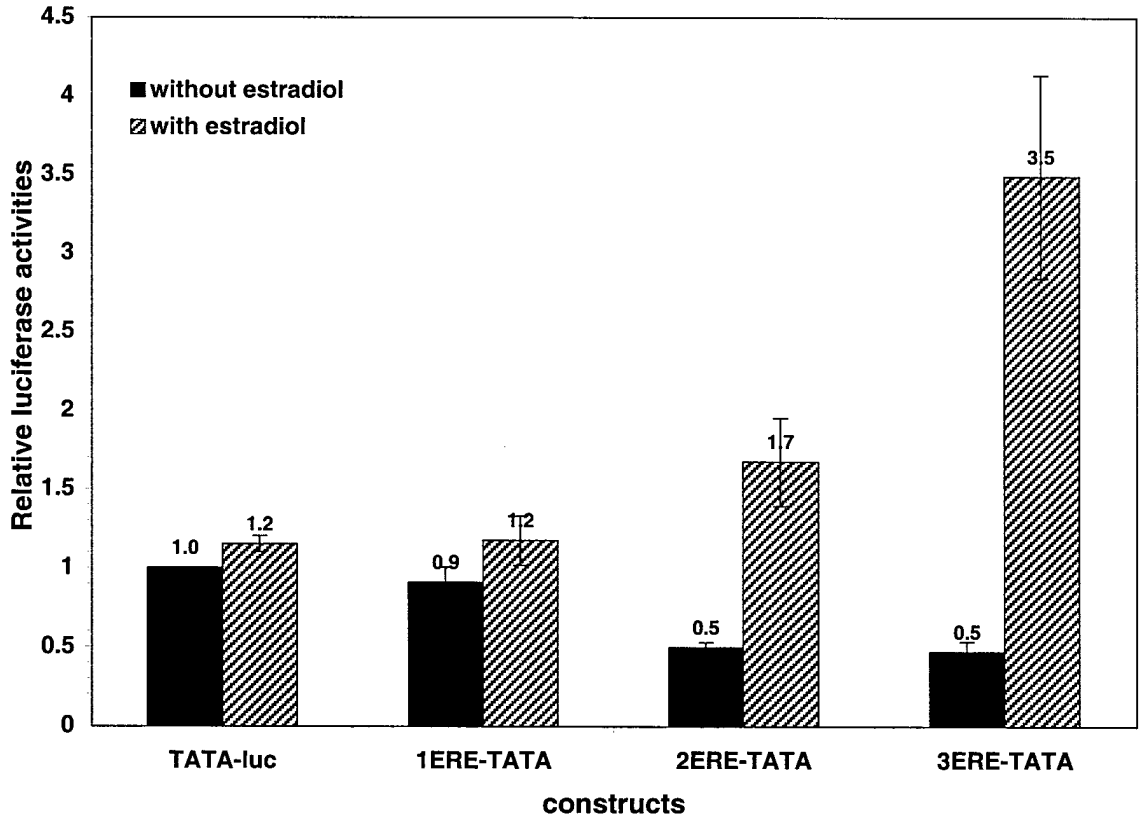
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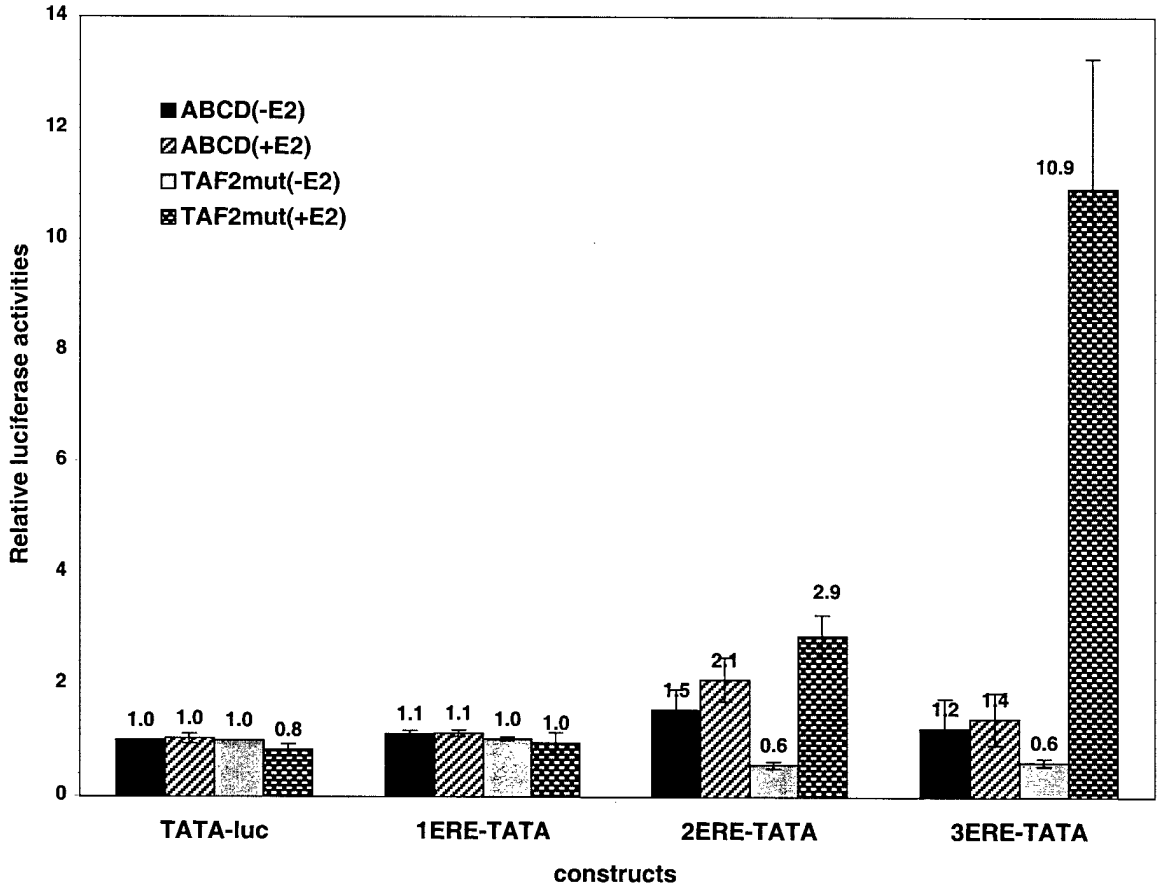
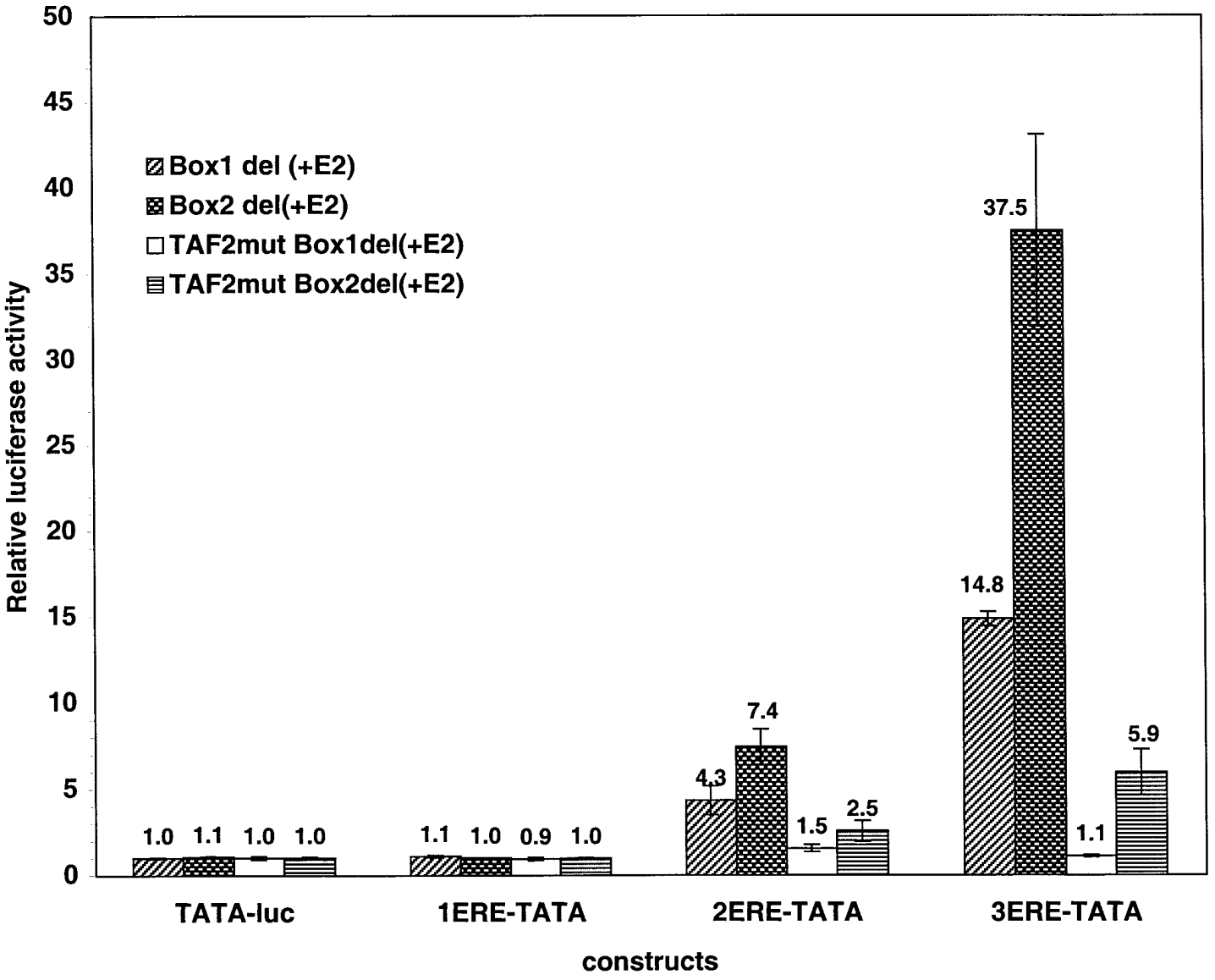
constructs


14
12
10
8
6
4
2
0
Relative luciferase activities
ABCD(-E2)
ABCD(+E2)
TAF2mut(-E2)
TAF2mut(+E2)
1.0
1.0
1.0
0.8
1.1
1.1
1.0
1.0
1.5
2.1
0.6
2.9
1.2
1.4
0.6
10.9
TATA-luc
1ERE-TATA
2ERE-TATA
3ERE-TATA
constructs


Box1 del (+E2)
Box2 del(+E2)
TAF2mut Box1del(+E2)
TAF2mut Box2del(+E2)
Relative luciferase activity
50
45
40
35
30
25
20
15
10
5
0
1.0 1.1 1.0 1.0
1.1 1.0 0.9 1.0
4.3
7.4
1.5
2.5
14.8
37.5
1.1
5.9
TATA-luc
1ERE-TATA
2ERE-TATA
3ERE-TATA
constructs